



## Research Paper

# Hydrogen peroxide derived from NADPH oxidase 4- and 2 contributes to the endothelium-dependent vasodilatation of intrarenal arteries



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## ABSTRACT

The role of NADPH oxidase (Nox)-derived reactive oxygen species in kidney vascular function has extensively been investigated in the harmful context of oxidative stress in diabetes and obesity-associated kidney disease. Since hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) has recently been involved in the non-nitric oxide (NO) non-prostanoid relaxations of intrarenal arteries, the present study was sought to investigate whether NADPH oxidases may be functional sources of vasodilator H<sub>2</sub>O<sub>2</sub> in the kidney and to assess their role in the endothelium-dependent relaxations of human and rat intrarenal arteries. Renal interlobar arteries isolated from the kidney of renal tumor patients who underwent nephrectomy, and from the kidney of Wistar rats, were mounted in microvascular myographs to assess function. Superoxide (O<sub>2</sub><sup>•-</sup>) and H<sub>2</sub>O<sub>2</sub> production was measured by chemiluminescence and Amplex Red fluorescence, and Nox2 and Nox4 enzymes were detected by Western blotting and by double immunolabeling along with eNOS. Nox2 and Nox4 proteins were expressed in the endothelium of renal arterioles and glomeruli co-localized with eNOS, levels of expression of both enzymes being higher in the cortex than in isolated arteries. Pharmacological inhibition of Nox with apocynin and of CYP 2C epoxygenases with sulfaphenazol, but not of the NO synthase (NOS), reduced renal NADPH-stimulated O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> production. Under conditions of cyclooxygenase and NOS blockade, acetylcholine induced endothelium-dependent relaxations that were blunted by the non-selective Nox inhibitor apocynin and by the Nox2 or the Nox1/4 inhibitors gp91ds-tat and GKT136901, respectively. Acetylcholine stimulated H<sub>2</sub>O<sub>2</sub> production that was reduced by gp91ds-tat and by GKT136901. These results suggest the specific involvement of Nox4 and Nox2 subunits as physiologically relevant endothelial sources of H<sub>2</sub>O<sub>2</sub> generation that contribute to the endothelium-dependent vasodilatation of renal arteries and therefore have a protective role in kidney vasculature.

## 1. Introduction

Oxidative stress and the associated endothelial dysfunction are key pathogenic factors underlying the vascular complications of metabolic disease including diabetic nephropathy [1,2]. However, reactive oxygen species (ROS) can act as both physiological and pathophysiological signaling molecules in the vascular wall. ROS like H<sub>2</sub>O<sub>2</sub> have

been involved in the endothelium-derived hyperpolarization (EDH) and vasodilation of arterioles from vascular beds such as the coronary circulation, wherein H<sub>2</sub>O<sub>2</sub> couples coronary blood flow to myocardium metabolism [3–5]. H<sub>2</sub>O<sub>2</sub> released from the endothelium by flow or agonists causes arterial relaxation through activation of K<sup>+</sup> channels and hyperpolarization of vascular smooth muscle (VSM) and by promoting Ca<sup>2+</sup> release from endothelial cell stores [4,6,7]. Sources of

*Abbreviations:* ACh, acetylcholine; COX, cyclooxygenase; CYP, cytochrome P450; EC, endothelial cell; EDH, endothelium-derived-hyperpolarization; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; Nox, NADPH oxidase enzymes; O<sub>2</sub><sup>•-</sup>, superoxide; Phe, phenylephrine; PSS, physiological saline solution; ROS, reactive oxygen species; SOD, superoxide dismutase; VSM, vascular smooth muscle

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vascular ROS include endothelial NOS (eNOS) [3], the mitochondria [8], the endothelial NADPH oxidases (Nox) [9], the cyclooxygenases (COX) [10] and the cytochrome P450 (CYP) epoxygenases [11].

The role of ROS in kidney vascular function has extensively been investigated in the context of harmful NADPH oxidase- and mitochondria-derived ROS generation and oxidative stress in diabetes, hypertension and obesity-associated kidney disease [1,12–16], but little is known about the involvement of species such as  $H_2O_2$  in the endothelial-dependent renal vasodilatation. NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular  $O_2$  through the Nox catalytic subunit to generate ROS and is the predominant source of renal superoxide ( $O_2^{\cdot-}$ ). In the kidney, NADPH oxidase enzymes (Nox) are widely distributed in renal vessels, glomeruli and nephron segments, Nox4 and also Nox2 being the predominant forms [17–19]. NADPH oxidase has been found to be a major source of ROS generation, oxidative stress and renal injury mostly under pathological conditions such as diabetic nephropathy and chronic kidney disease [15,16,19]. However, Nox isoforms have also been involved in kidney physiological processes such as regulation of glucose production and handling in the proximal tubule, tubule-glomerular feed-back in macula densa, regulation of renal tubule electrolyte transport and regulation of afferent arteriole responsiveness [18,20,21].

We have recently demonstrated that endothelial-derived  $H_2O_2$  is involved in the non-nitric oxide (NO) non-prostanoid EDH relaxations of rat intrarenal arteries through various mechanisms including stimulation of endothelial  $K^+$  channels and hyperpolarization, and  $Ca^{2+}$ -dependent and  $Ca^{2+}$  sensitization mechanisms in VSM [22]. Moreover, in our study CYP epoxygenases were identified as relevant enzymatic sources of  $H_2O_2$  generation in the renal endothelium under physiological conditions. Nox2 and Nox4 mRNA have been identified in intrarenal arteries of the human kidney although their functional significance and the role of Nox subunits in renal hemodynamics remain unclear [18]. Furthermore, the predominant Nox isoform in the kidney, Nox4, can have potential beneficial effects on vasodilator function [23]. Therefore, the present study was designed to investigate whether NADPH oxidase may be a relevant functional source of vasodilator  $H_2O_2$  in the renal endothelium and assess its role in the endothelium-dependent vasodilation of human and rat intrarenal arteries.

## 2. Materials and methods

### 2.1. Animal model

In the present study, 12–14 weeks old male Wistar rats were housed at the Pharmacy School animal care facility and maintained on standard chow and water ad libitum. All animal care and experimental protocols conformed to the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and were approved by the Institutional Animal Care and Use Committee of Madrid Complutense University. Animals were killed and the kidneys quickly removed and placed in cold ( $4^\circ C$ ) physiological saline solution (PSS) of the following composition (mM): NaCl 119,  $NaHCO_3$  25, KCl 4.7,  $KH_2PO_4$  1.17,  $MgSO_4$  1.18,  $CaCl_2$  1.5, EDTA 0.027 and glucose 11, continuously gassed with a mixture of 5%  $CO_2$ /95%  $O_2$  to maintain pH at 7.4.

### 2.2. Patients

Human renal tissues were obtained from renal tumour patients who underwent nephrectomy. The investigation with human tissue conformed to the principles outlined in the Declaration of Helsinki. Permission was obtained from the Ethics Committee of the University Hospital Puerta de Hierro-Majadahonda, Spain (Reg. no 5.16) and patients gave their informed consent. We investigated tissues from 9 patients (2 female, 7 males) with a mean  $\pm$  SEM age of  $65 \pm 3$  years. Out of these patients, 1 was diagnosed with diabetes mellitus and 1 with

hydronephrosis and kidneys were discarded from the study. Plasma creatinine levels were  $1.02 \pm 0.20$  mg/ml ( $n = 7$ ).

### 2.3. Dissection and mounting of microvessels

Tissue samples from tumour-free parts of the kidney were obtained after kidney resection in the Pathology laboratory, placed in ice-cold physiological salt solution (PSS) and taken to the laboratory for dissection. Microdissection under the microscope of tumor-free kidney samples from 7 patients containing renal cortex and medulla, yielded 20 distal interlobar and arcuate arteries. Small samples of both renal arteries and cortex were also dissected out for ROS measurements.

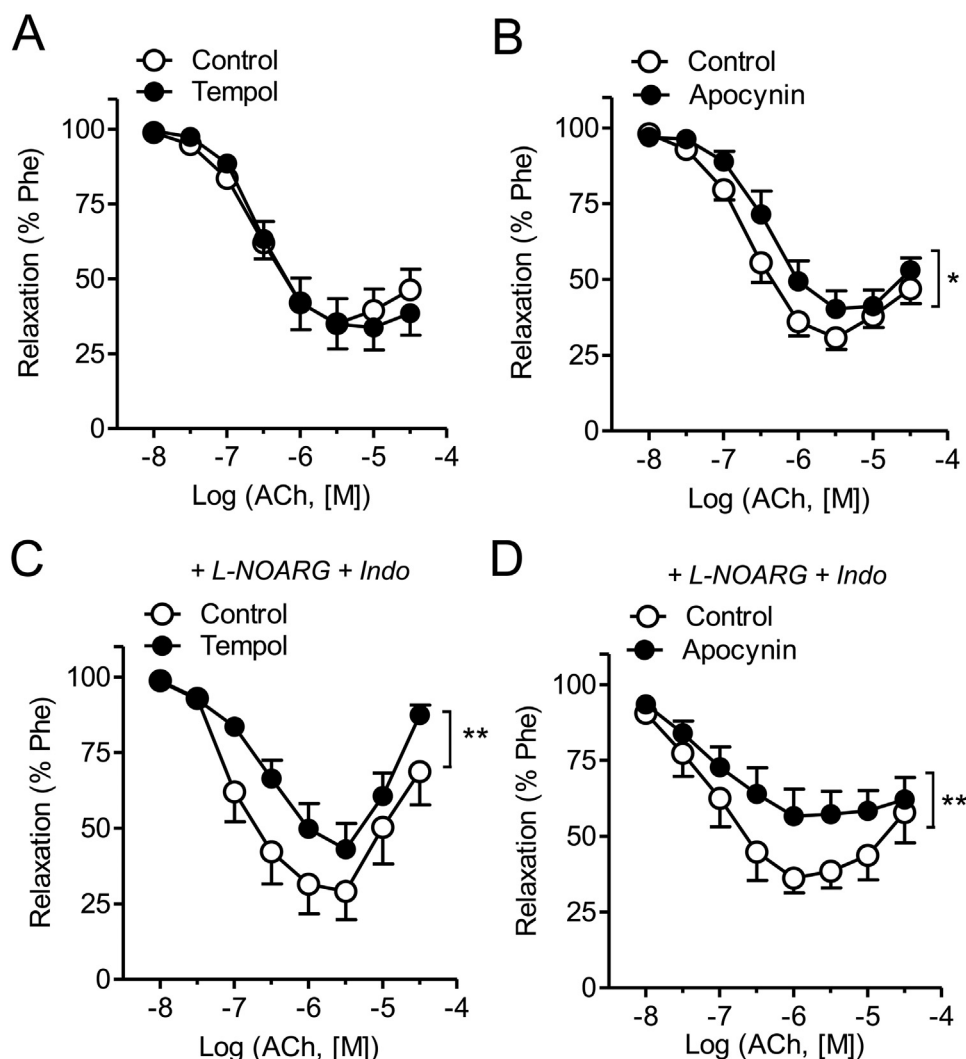
Renal interlobar arteries, second- or third order branches of the renal artery from Wistar rats, were carefully dissected by removing the medullary connective tissue and mounted in parallel in double microvascular myographs (Danish Myotechnology, Denmark) by inserting two  $40 \mu m$  tungsten wires into the vessel lumen. After mounting the arteries were equilibrated for 30 min in PSS maintained at  $37^\circ C$ . The relationship between passive wall tension and internal circumference was determined for each individual artery and from this, the internal circumference,  $L_{100}$  corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel *in situ* was calculated. The arteries were set to an internal diameter  $l_1$  equal to 0.9 times  $l_{100}$  ( $l_1 = 0.9 \times l_{100}$ ), since force development in intrarenal arteries is close to maximal at this internal lumen diameter [10].

### 2.4. Experimental procedures for the functional experiments

At the beginning of each experiment, arteries were challenged twice with 120 mM  $K^+$  solution (KPSS) in order to test vessel viability. Endothelium-dependent vasodilatation of renal arteries was assessed by the relaxant effects of acetylcholine (ACh) upon addition of cumulative concentrations of this agent on arteries precontracted with phenylephrine (Phe) and previously incubated with L-NOARG (100  $\mu M$ ) and indomethacin (0.3  $\mu M$ ) to block NOS and COX enzymes, respectively. The responses to exogenous ACh were further obtained in the absence and presence of the SOD scavenger tempol (30  $\mu M$ ), the specific mitochondrial superoxide scavenger MitoTEMPO (1  $\mu M$ ), the non-selective Nox inhibitors apocynin (30  $\mu M$ ) and plumbagin (1  $\mu M$ ), the dual Nox1/4 inhibitor GKT137831 (0.1  $\mu M$ ) and the Nox2 inhibitor Nox2ds-tat (1  $\mu M$ ) [24]. The drugs were added to the myograph chamber 30 min before a second concentration-response curve was performed, and the Phe concentration was adjusted to match the contraction during the first control curve assessment.

### 2.5. Measurement of superoxide production by chemiluminescence

Changes in basal and NADPH-stimulated levels of  $O_2^{\cdot-}$  were detected by lucigenin-enhanced chemiluminescence, as previously described [10,22]. Cortex samples and 4–6 segments of the renal interlobar arteries about 4–5 mm long from each patient or Wistar rat were dissected and equilibrated in PSS for 30 min at room temperature and then incubated in the absence (controls) and presence of the NADPH oxidase inhibitors plumbagin (1  $\mu M$ ), Nox2ds-tat (1  $\mu M$ ) and GKT136901 (0.1  $\mu M$ ) for 30 min at  $37^\circ C$ , and then stimulated with NADPH (100  $\mu M$ ) which was added 15 min previous to ROS measurements. Samples were then transferred to microtiter plate wells containing 5  $\mu M$  bis-N-methylacridinium nitrate (lucigenin) in the absence and presence of different ROS inhibitors. Chemiluminescence was measured in a luminometer (BMG Fluostar Optima), and for calculation baseline values were subtracted from the counting values under the different experimental conditions and superoxide production was normalized to dry tissue weight.



**Fig. 1.** Effect of Nox inhibition on the non-NO non-prostanoid endothelial relaxations of rat intrarenal arteries. (A–D) Effects of the SOD mimetic tempol (30  $\mu$ M) and the Nox oxidase inhibitor apocynin (30  $\mu$ M) on the relaxant responses to ACh of rat renal interlobar arteries (A, B) in the absence of L-NOARG (100  $\mu$ M) and indomethacin (0.3  $\mu$ M) (Indo) to inhibit NO and prostanoid synthesis, or (C, D) under conditions of NOS and COX blockade. Results are expressed as percentage of the precontraction induced by phenylephrine (Phe). Data are shown as the mean  $\pm$  SEM of 7–8 arteries (4 animals). Significant differences were analyzed by paired Student *t*-test. \**p* < 0.05; \*\**p* < 0.01 versus control before treatment.

**Table 1**  
Effect of Nox inhibitors, of the SOD mimetic tempol and of the mitochondrial ROS scavenger mitoTEMPO on the non-NO non-prostanoid endothelium-dependent vasodilatation of rat intrarenal arteries.

	ACh			<i>I</i> <sub>1</sub>
	<i>pEC</i> <sub>50</sub>	<i>E</i> <sub>max</sub>	<i>n</i>	
Control	6.85 $\pm$ 0.10	71 $\pm$ 9	7	303 $\pm$ 16
+ Tempol (30 $\mu$ M)	6.54 $\pm$ 0.17	57 $\pm$ 8 <sup>b</sup>	7	303 $\pm$ 16
Control	7.08 $\pm$ 0.20	76 $\pm$ 5	8	293 $\pm$ 33
+ Apocynin (30 $\mu$ M)	7.10 $\pm$ 0.20	48 $\pm$ 8 <sup>b</sup>	8	293 $\pm$ 33
Control	6.77 $\pm$ 0.21	68 $\pm$ 3	11	262 $\pm$ 15
+ GKT136901 (0.1 $\mu$ M)	6.69 $\pm$ 0.09	42 $\pm$ 5 <sup>c</sup>	11	262 $\pm$ 15
Control	6.88 $\pm$ 0.17	64 $\pm$ 5	6	250 $\pm$ 25
+ Plumbagin (1 $\mu$ M)	–	33 $\pm$ 3	6	250 $\pm$ 25
Control	6.47 $\pm$ 0.34	67 $\pm$ 5	8	293 $\pm$ 13
+ Nox2ds-tat (1 $\mu$ M)	6.61 $\pm$ 0.29	50 $\pm$ 8 <sup>b</sup>	8	293 $\pm$ 13
Control	6.68 $\pm$ 0.21	72 $\pm$ 8	8	292 $\pm$ 15
+ MitoTEMPO (1 $\mu$ M)	6.25 $\pm$ 0.58	50 $\pm$ 7 <sup>c</sup>	8	292 $\pm$ 15

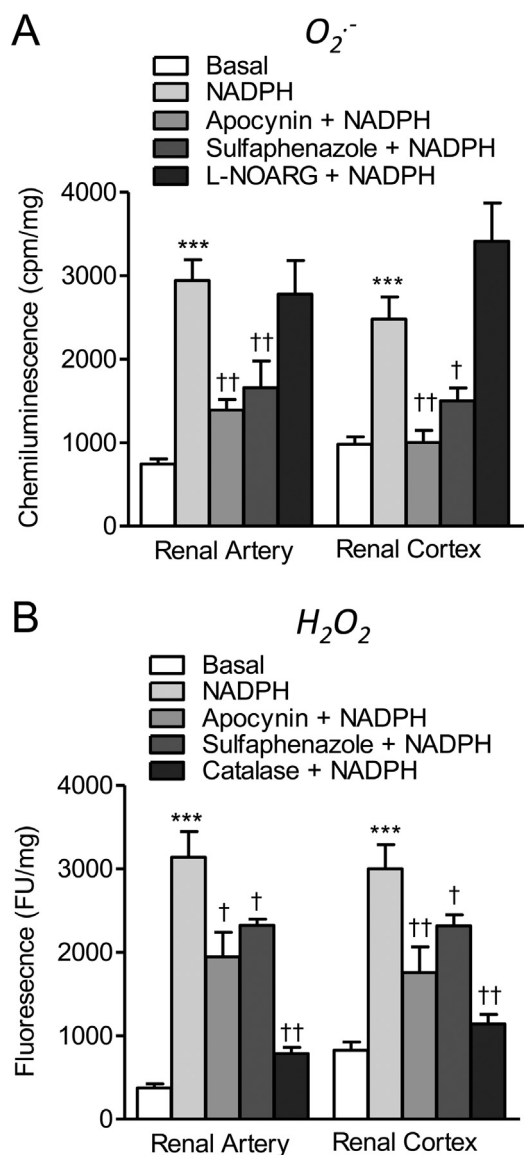
Values represent mean  $\pm$  S.E.M. of the number *n* of individual arteries, 1–2 per animal. *pEC*<sub>50</sub> is  $-\log EC_{50}$ , *EC*<sub>50</sub> being the agonist concentration giving half-maximal relaxation; *E*<sub>max</sub> = maximal relaxation (% Phe). *I*<sub>1</sub> is the effective lumen diameter of the arteries. Significant differences were analyzed by paired Student *t*-test. <sup>a</sup>*p* < 0.05; <sup>b</sup>*p* < 0.01; <sup>c</sup>*p* < 0.001, versus control before treatment

2.6. Measurement of hydrogen peroxide by Amplex Red

H<sub>2</sub>O<sub>2</sub> production was measured by Amplex Red H<sub>2</sub>O<sub>2</sub> assay Kit (Life Technologies) in renal arteries and cortex [22]. Samples were equilibrated in HEPES-physiological saline solution (PSS) for 30 min at room temperature and then incubated in the absence (controls) and presence of the Nox inhibitors plumbagin (1  $\mu$ M), Nox2ds-tat (1  $\mu$ M), GKT136901 (0.1  $\mu$ M) or catalase (200 U/ml) for 30 min at 37 °C. Arteries and cortex samples were then transferred to microtiter plate black wells containing 10 mM final concentration (Amplex Red) and 10 U/ml final concentration (horseradish peroxidase) in the absence and presence of different ROS sources inhibitors and some samples were stimulated with either NADPH (100  $\mu$ M) 15 min or ACh (10  $\mu$ M) just prior to determination. Fluorescence was measured in a fluorimeter (BMG Fluostar Optima), using an excitation filter of 544 nm and an emission filter of 590 nm. Background fluorescence was subtracted from the counting values under the different experimental conditions and H<sub>2</sub>O<sub>2</sub> production was normalized to dry tissue weight.

2.7. Western blotting analysis of Nox subunits

Interlobar arteries and renal cortex samples from Wistar rats were homogenized on ice in lyses buffer containing 10 mM Tris-HCl (pH 7.4), 1% SDS, 1 mM sodium vanadate and 0.01% protease inhibitor cocktail (Sigma Aldrich, Madrid, Spain). After centrifugation at 15000  $\times$  g for 20 min at 4 °C, proteins in the supernatants were quantified by the DC



**Fig. 2.** NADPH-dependent ROS generation and effect of inhibition of Nox, CYP 2C epoxygenase and NOS. (A, B) Basal and NADPH-stimulated  $O_2^{\cdot -}$  (A) and  $H_2O_2$  (B) production in rat renal interlobar arteries and renal cortex measured by lucigenin-enhanced chemiluminescence and Amplex Red fluorescence assays, respectively. The effect of inhibition of Nox with apocynin (30  $\mu$ M), of CYP 2C epoxygenases with sulfaphenazole (1  $\mu$ M) and of eNOS with L-NOARG (100  $\mu$ M) was assessed on NADPH (100  $\mu$ M)-dependent  $O_2^{\cdot -}$  (A) and  $H_2O_2$  (B) production. Results are expressed as counts per minute (cpm) per mg of tissue for chemiluminescence and relative fluorescence units (RFU) per mg of tissue. Bars represent mean  $\pm$  SEM of 8–10 arterial or cortical samples from 4–5 animals. Statistically significant differences were calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test \*\*\* $p < 0.001$  versus basal levels. †  $p < 0.05$ ; ††  $p < 0.05$  versus NADPH-stimulated.

Protein Assay Kit (Bio-Rad, Madrid Spain). For each sample, 30–80  $\mu$ g protein/lane was separated in a 10% polyacrylamide gel (SDS-PAGE) and, after migration, were transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, Madrid, Spain). For immunodetection, membranes were incubated overnight at 4 °C with the polyclonal primary antibodies: anti-Nox2/gp91phox (1/1000), (Abcam, Cambridge, UK), anti-Nox4 (1/500) and anti-MnSOD (1/1000) (Santa Cruz Biotechnology, Quimigen, Madrid, Spain). The blots were also probed for  $\beta$ -actin (1/40,000) as the loading control using a mouse monoclonal antibody (Sigma Aldrich, Madrid, Spain). The membranes were then washed in 0.05% Tween-20, incubated with HRP-conjugated

secondary antibodies for 1h at room temperature, and then washed and visualized by chemiluminescence (ECL Select-kit, GE Healthcare, Madrid, Spain) on ImageQuant LAS 500 imaging system (GE Healthcare, Madrid, Spain). Relative levels of immunoreactive proteins were quantified using Quantity One software (Bio-Rad Laboratories, Madrid, Spain). Densitometry units were normalized to  $\beta$ -actin.

## 2.8. Immunohistochemistry

Tissue samples from kidneys containing interlobar arteries and samples of renal cortex from Wistar rats were immersion-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate-buffer (PB), cryoprotected in 30% sucrose in PB and snap-frozen in liquid nitrogen and stored at -80 °C. Transversal sections 5  $\mu$ m thick were obtained by means of a cryostat and preincubated in 10% normal goat serum in PB containing 0.3% Triton-X-100 for 2–3 h. Nox enzymes expression was determined by immunofluorescence by incubating renal sections from Wistar rats with polyclonal primary antibodies: anti-Nox2/gp91phox (1/50), (Abcam, Cambridge, UK) and anti-Nox4 (1/100) (Santa Cruz Biotechnology, Quimigen, Madrid, Spain), and a mouse monoclonal anti endothelial NO synthase (eNOS) (Abcam, Cambridge, UK) diluted at 1:400 for 48 h, washed and allowed to react with a goat secondary serum (Chemicon International Inc) (anti-rabbit for the Nox2 and for the Nox4) diluted 1:200 for 3 h at room temperature. Secondary antibodies used were Alexa Fluor 594 (red) and Alexa Fluor 488 (green). The slides were covered with a specific medium containing DAPI, which stains all cell nuclei. The observations were made with a fluorescence microscope (Olympus IX51). No immunoreactivity could be detected in sections incubated in the absence of the primary antisera. Preadsorption with Nox proteins showed no cross-reactivity to the antibodies.

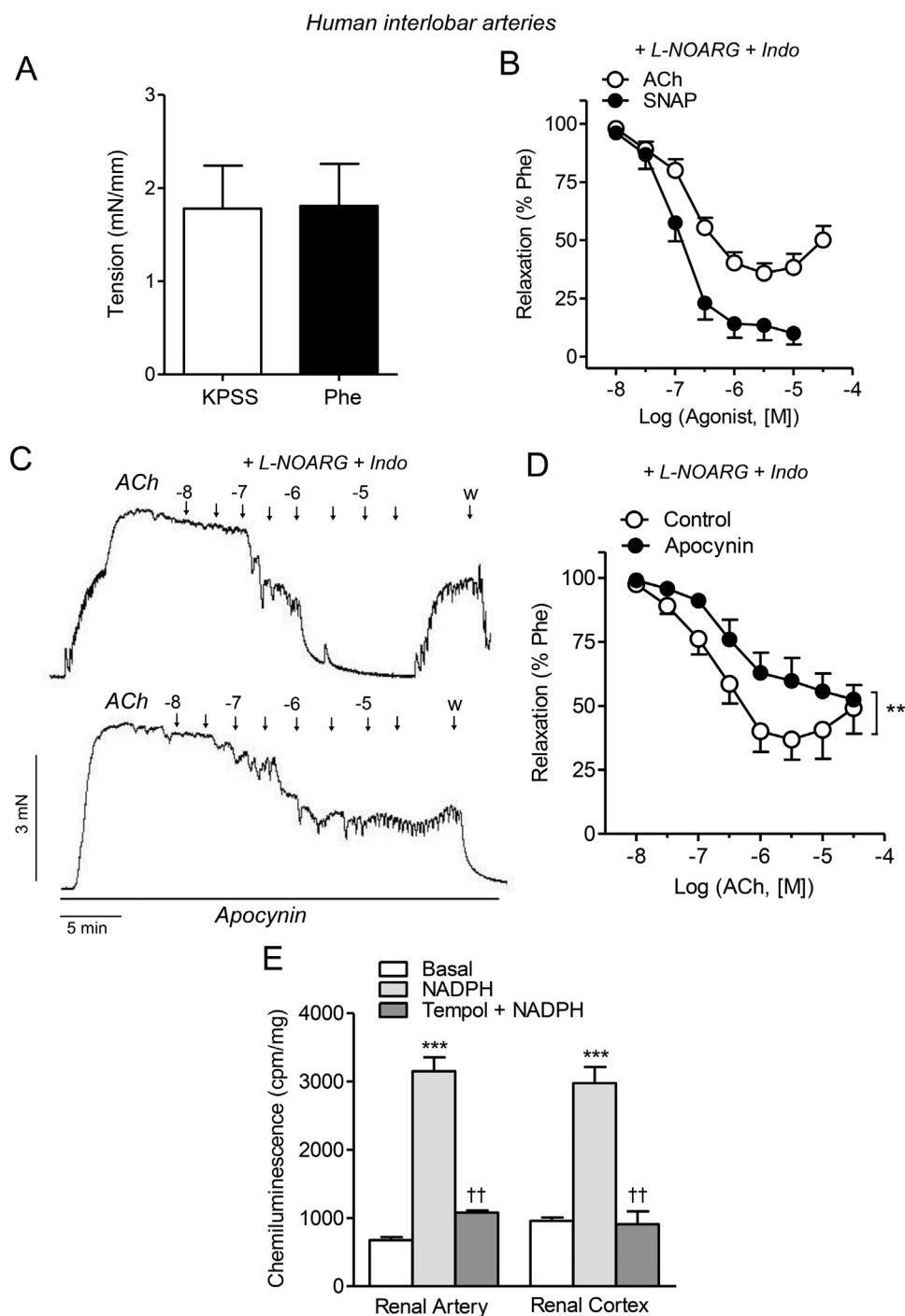
## 2.9. Data presentation and statistical analysis

For the functional experiments results are expressed as either  $Nm^{-1}$  of tension or as a percent of the responses to either Phe or KPSS in each artery, as means  $\pm$  SEM of 6–15 arteries (1–2 from each patient or animal) for the functional experiments. For the measurement of  $O_2^{\cdot -}$  or  $H_2O_2$  production, results are expressed in counts per minute (cpm) per mg of tissue and relative fluorescence units (RFU) per mg of tissue in arterial segments and cortex sample, respectively, as means  $\pm$  SEM of 3–7 patients or means  $\pm$  SEM of 4–7 animals. The statistical differences between means were analyzed by using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons involving more than two groups or by paired or unpaired Student's *t*-test for comparison between two groups. Probability levels lower than 5% were considered significant. All calculations were made using a standard software package (Prism 5.0, GraphPad, San Diego, CA).

## 3. Results

### 3.1. ROS scavenging and Nox inhibition reduce non-NO non-prostanoid endothelium-dependent relaxations of intrarenal arteries

The role of Nox-derived ROS in the endothelium-dependent vasodilatation of intrarenal arteries was initially assessed by the effects of the ROS scavenger tempol and of the non-selective Nox inhibitor apocynin on the relaxant responses to the endothelial agonist ACh. While none or a minor inhibitory effect was found for either tempol or apocynin on the endothelial relaxations of rat interlobar arteries (Fig. 1A–B), a marked inhibitory action of both compounds in the relaxations induced by ACh was unmasked when assessing relaxations under conditions of NOS and COX enzymes blockade (Fig. 1C, D; Table 1). Basal  $O_2^{\cdot -}$  and  $H_2O_2$  production assessed by lucigenin chemiluminescence and Amplex Red fluorescence assays, respectively, was markedly enhanced by NADPH in both renal interlobar arteries and renal cortex from rat kidney (Fig. 2A, B). Blockade of Nox with



**Fig. 3.** Nox-derived ROS are involved in the non-NO non-prostanoid endothelium-dependent relaxations of human intrarenal arteries. (A) Contractile responses elicited by depolarization with 125 mM K<sup>+</sup> solution (KPSS) and by the  $\alpha_1$ -adrenoceptor agonist phenylephrine (Phe, 50  $\mu$ M) (B) Relaxant responses elicited by the endothelial agonist acetylcholine (ACh) under conditions of NOS and COX blockade (100  $\mu$ M L-NOARG and 0.3  $\mu$ M indomethacin, Indo) to inhibit NO and prostanoid synthesis, compared to the endothelium-independent NO donor SNAP in human distal interlobar and arcuate arteries precontracted with Phe. Data are shown as the mean  $\pm$  SEM of 7–14 arteries from 7 patients. (C) Representative traces of a human arcuate artery with an effective lumen diameter  $l_1 = 377 \mu$ m showing the non-NO non-prostanoid relaxant responses to ACh in the absence (upper trace) and the presence of the Nox inhibitor apocynin (30  $\mu$ M) (lower trace). (D) Average inhibitory effect induced by apocynin on the relaxations elicited by ACh in human intrarenal arteries. Data represent mean  $\pm$  SEM of 7 arteries from 3 patients. (E) Basal and NADPH-stimulated O<sub>2</sub><sup>-</sup> production in samples of human intrarenal arteries and renal cortex measured by lucigenin-enhanced chemiluminescence and blunting effect of the SOD scavenger tempol (30  $\mu$ M). Bars show mean  $\pm$  SEM 6–10 arterial or cortical samples from 3 patients. Significant differences were analyzed by paired Student *t*-test. \*\**p* < 0.01 versus control before treatment (D) and by one way ANOVA \*\*\**p* < 0.001 versus basal levels. †† *p* < 0.05 versus NADPH-stimulated.

apocynin or CYP 2C epoxygenases with sulfaphenazole, had an inhibitory effect on NADPH chemiluminescent and fluorescent signals which suggests that both Nox and CYP 2C enzymes are involved in NADPH-dependent O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> production in renal tissues [22]. However, the lack of inhibitory effect of L-NOARG on NADPH-dependent chemiluminescent signals initially rules out a role for eNOS in ROS generation under physiological conditions in intrarenal arteries (Fig. 2A).

### 3.2. Non-NO non-prostanoid endothelium-dependent relaxations of human intrarenal arteries involve Nox-derived ROS

Human distal interlobar and arcuate arteries dissected out from tumor-free kidney samples obtained from 7 patients, had internal

lumen diameters ( $l_1$ ) ranging between 170–670  $\mu$ m ( $428 \pm 58 \mu$ m,  $n = 18$ ). Average vasoconstrictor responses and non-NO non-prostanoid endothelium-dependent relaxations of human intrarenal arteries are shown in Fig. 3A and B. Depolarization with high K<sup>+</sup> solution (KPSS) and  $\alpha_1$ -adrenoceptor stimulation with 50  $\mu$ M Phe elicited sustained contractions of similar magnitude (Fig. 3A). NO-mediated endothelium-dependent relaxations have earlier been shown in human intrarenal arteries [18]. In order to assess the involvement of ROS in the non-NO non-prostanoid endothelial vasodilatation, relaxations to ACh were performed under conditions of NOS and COX blockade. In Phe-precontracted arteries treated with L-NOARG and indomethacin, ACh elicited non-NO no-prostanoid concentration-dependent relaxations, pD<sub>2</sub> values and maximal responses being  $6.75 \pm 0.12$  and  $65 \pm 5\%$  ( $n = 13$ ), respectively (Fig. 3B). Stimulation of the arteries with the

**Table 2**  
Effect of Nox inhibitors on the non-NO non-prostanoid endothelium-dependent vasodilatation of human intrarenal arteries.

	ACh		n	I <sub>1</sub>
	pEC <sub>50</sub>	E <sub>max</sub>		
Control	6.71 ± 0.18	63 ± 8	7	282 ± 58
+ Apocynin (30 μM)	6.24 ± 0.24 <sup>b</sup>	48 ± 6 <sup>b</sup>	7	282 ± 58
Control	6.84 ± 0.12	70 ± 6	9	442 ± 68
+ GKT136901 (0.1 μM)	6.52 ± 0.10 <sup>a</sup>	35 ± 7 <sup>c</sup>	9	442 ± 68
Control	6.44 ± 0.11	69 ± 1	6	592 ± 99
+ Nox2ds-tat (1 μM)	6.17 ± 0.19 <sup>b</sup>	35 ± 14 <sup>a</sup>	6	592 ± 99

Values represent mean ± S.E.M. of the number *n* of individual arteries, 1–2 from each patient (3–4 patients). pEC<sub>50</sub> is -logEC<sub>50</sub>, EC<sub>50</sub> being the agonist concentration giving half-maximal relaxation; E<sub>max</sub> = maximal relaxation (% Phe). I<sub>1</sub> is the effective lumen diameter of the arteries. Significant differences were analyzed by paired Student *t*-test. <sup>a</sup>p < 0.05; <sup>b</sup>p < 0.01; <sup>c</sup>p < 0.001, versus control before treatment.

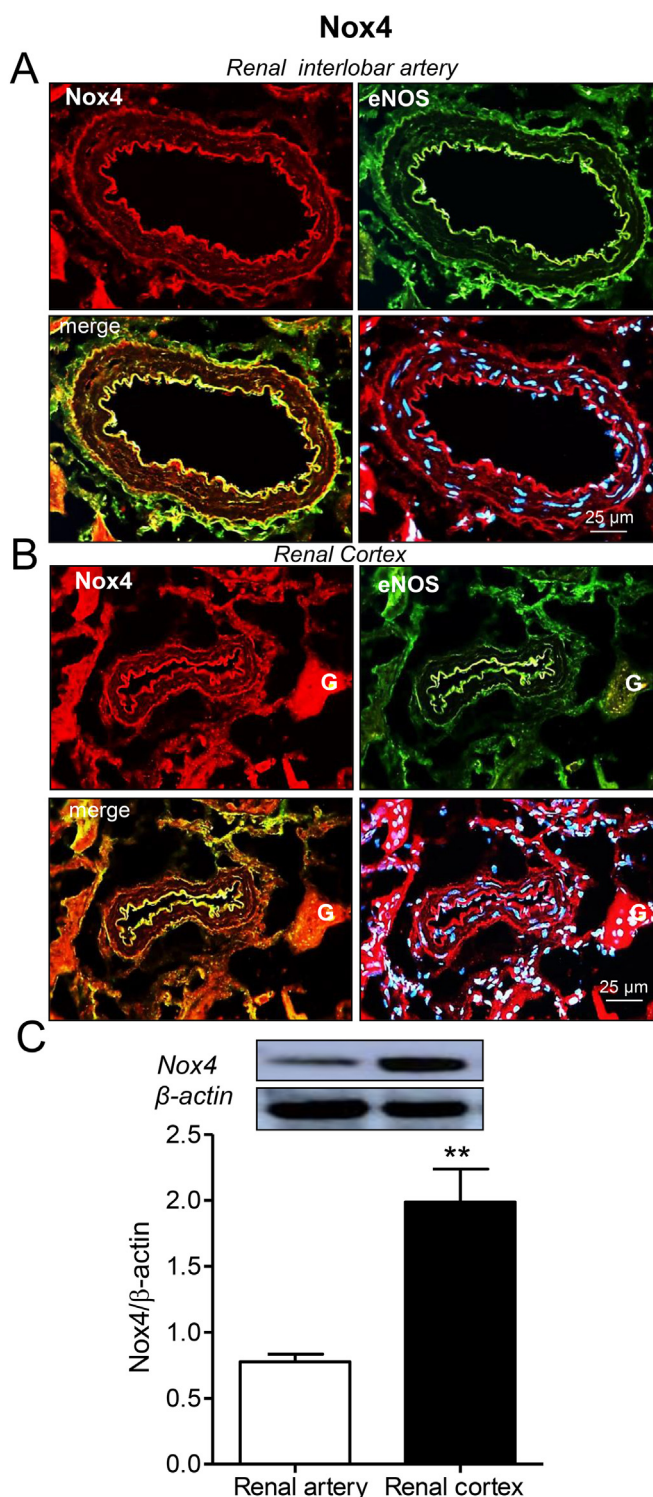
endothelium-independent NO donor SNAP maximally relaxed human intrarenal arteries precontracted with Phe (pD<sub>2</sub> 7.02 ± 0.08 and E<sub>max</sub> 90 ± 5%, n = 6) (Fig. 3B).

In order to assess whether Nox is a functionally relevant source of vasodilator ROS in human intrarenal arteries, the effect of apocynin was tested on the relaxations to ACh under conditions of NOS and COX blockade. Apocynin significantly reduced endothelium-dependent ACh relaxant responses (Fig. 3C–D; Table 2). Basal O<sub>2</sub><sup>•-</sup> levels in renal arteries and cortex were markedly enhanced to similar levels upon stimulation with NADPH, and chemiluminescent signals were blunted by the SOD mimetic tempol (Fig. 3E), thus suggesting NADPH-dependent ROS generation in human vascular and cortical tissues.

### 3.3. Nox4 is involved in the endothelium-dependent relaxations and ROS generation of rat intrarenal arteries

Nox4 and Nox2 but not Nox1 proteins have been identified in human intrarenal arteries [18]. To further assess which Nox catalytic subunit is involved in ROS-mediated endothelial vasodilatation of renal arteries, Nox expression was determined in rat renal interlobar arteries compared to kidney cortex samples, and the effects of Nox inhibitors were evaluated on ROS production and on the relaxant responses to the endothelial agonist ACh. Nox4 is abundant in kidney cortex [25] and immunostaining of arterial sections with Nox4 antibodies revealed that this subunit was uniformly distributed in the endothelium of rat renal arteries colocalized with eNOS and in VSM (Fig. 4A). In samples of renal cortex, Nox4 was found in renal tubules but also in vascular tissue, in the glomeruli and the endothelial lining of arterioles (Fig. 4B). Western blot analysis confirmed that Nox4 protein levels were abundant and significantly higher in rat renal cortex compared to isolated renal arteries (Fig. 4C).

The effect of Nox4 inhibitors was examined on ROS production of kidney tissues. The selective Nox1/4 inhibitor GKT136901, and the non-selective Nox4 inhibitor plumbagin reduced both O<sub>2</sub><sup>•-</sup> (Fig. 5A) and H<sub>2</sub>O<sub>2</sub> (Fig. 5B) formation stimulated by NADPH in samples of rat renal interlobar arteries and renal cortex. Both compounds had a larger inhibitory effect on O<sub>2</sub><sup>•-</sup> production in rat renal interlobar arteries, while the inhibitory effect on H<sub>2</sub>O<sub>2</sub> generation was higher in renal cortex samples (Fig. 5A, B). We further evaluated whether Nox4 is a functional source of endothelial vasodilator ROS in intrarenal arteries. Both the relaxant responses and the H<sub>2</sub>O<sub>2</sub> production elicited by ACh under conditions of NOS and COX blockade were markedly inhibited by GKT136901 (Fig. 6A–C; Table 1), and also by plumbagin (Fig. 6D). The two compounds also reduced the ACh endothelial relaxations in the absence of L-NOARG and indomethacin (Fig. 6E, F). Collectively these findings suggest that endothelial Nox4-derived H<sub>2</sub>O<sub>2</sub> is involved in vasodilatation of renal arteries.



(caption on next page)

Since Nox4 has been identified as a source of ROS generation in the mitochondria, the effect of the mitochondrial ROS scavenger MitoTEMPO was evaluated on the relaxant responses to ACh. MitoTEMPO mimicked the inhibitory effect of Nox4 inhibitors on the endothelium-dependent vasodilator responses of intrarenal arteries (Fig. 7A, B; Table 1). On the other hand, Western blot analysis demonstrated that protein levels of the mitochondrial MnSOD, catalyzing enzyme that dismutates O<sub>2</sub><sup>•-</sup> to H<sub>2</sub>O<sub>2</sub>, were high in renal arteries and similar to those in the renal cortex (Fig. 7C), thus ensuring the

**Fig. 4.** Nox4 is expressed in the endothelium of renal arteries and arterioles colocalized with eNOS. (A, B) Immunohistochemical demonstration of Nox4 in renal interlobar artery and cortex from rat kidney. Immunofluorescence for Nox4 protein (red areas) was distributed throughout the endothelial lining of the renal interlobar artery and in VSM (A) and in the glomeruli (G) and endothelium of arterioles in the renal cortex (B). Endothelial cell layer was visualized with the anti-eNOS marker (green). Immunofluorescence double labelling for eNOS marker and Nox4 expression demonstrates colocalization in renal endothelium (yellow areas) in both renal artery and cortex. Scale bars indicate 25  $\mu\text{m}$ . Sections are representative of  $n = 3$  animals. (c) Western blot analysis of Nox4 expression in samples of renal artery and cortex showing that Nox4 protein levels were higher in samples of renal cortex than in those of renal arteries. Results were quantified by densitometry and presented as a ratio of density of Nox4 band vs those of  $\beta$ -actin from the sample. Data are shown as the mean  $\pm$  SEM of 4 animals. Significant differences between means were analyzed by using unpaired  $t$ -test  $^{**}P < 0.01$  versus renal cortex.

generation of  $\text{H}_2\text{O}_2$  from NADPH oxidase-derived mitochondrial  $\text{O}_2^{\cdot-}$  in the arterial wall.

### 3.4. Role of Nox2 in the endothelium-dependent relaxations and ROS generation of intrarenal arteries

Nox2 has been involved in the regulation of myogenic vasoconstrictor responses of renal arterioles [21]. The expression and activity of Nox2 and its involvement in the endothelial vasodilator function of renal arteries were assessed. Immunoreaction for Nox2 was found in both endothelium and VSM of interlobar arteries (Fig. 8A), while in renal cortex Nox2 was widely distributed in tubular structures but also in the endothelium of small cortex arterioles wherein Nox2 was colocalized with eNOS (Fig. 8B). Western blot analysis showed Nox2 protein levels in rat intrarenal arteries that averaged about half of those in renal cortex (Fig. 8C). The selective inhibitor of Nox2 Nox2ds-tat had a pronounced inhibitory effect on both  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  production stimulated by NADPH in renal interlobar arteries but not in renal cortex (Fig. 9A, B).

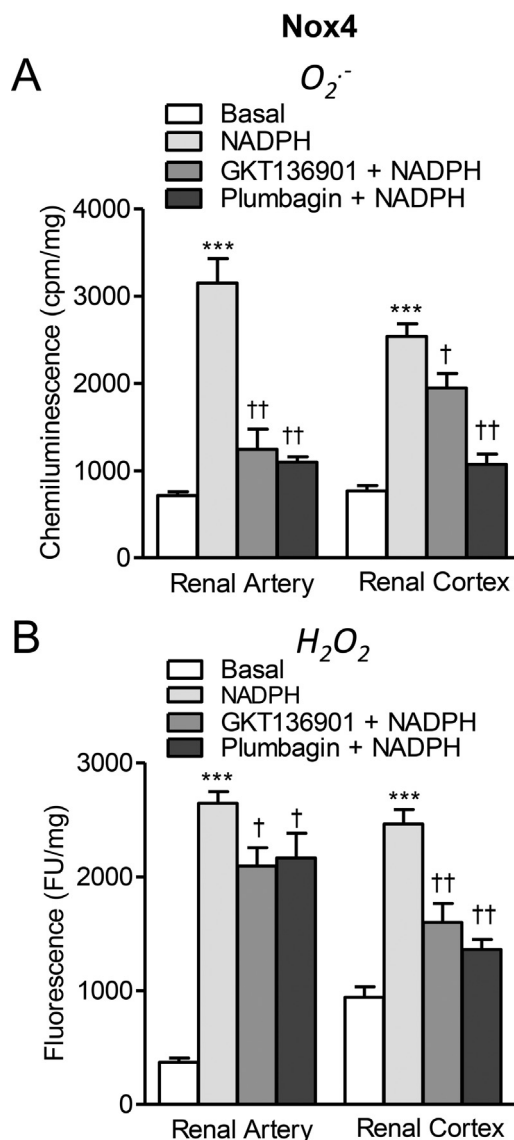
To assess whether Nox2 may also contribute to the renal endothelium-dependent vasodilatation, renal arteries were treated with the selective Nox2 inhibitor Nox2ds-tat. Under conditions of NOS and COX blockade, Nox2ds-tat treatment significantly reduced both the relaxant responses (Fig. 10A, B; Table 1) and the  $\text{H}_2\text{O}_2$  production (Fig. 10C) stimulated by the endothelial agonist ACh. Nox2 inhibition also changed the sustained  $\text{H}_2\text{O}_2$ -mediated relaxations into more EDH-type phasic relaxant responses mediated by  $\text{K}^+$  channels (Fig. 10A).

### 3.5. Effect of Nox4 and Nox2 inhibitors on the non-NO non-prostanoid endothelium-dependent relaxations and ROS generation of human intrarenal arteries

Involvement Nox4 and Nox2 in the endothelium-dependent vasodilation of human intrarenal arteries was further investigated by testing the effect of the inhibitors GKT136901 and Nox2ds-tat on the ACh relaxant responses and ROS generation under conditions of NOS and COX blockade. Both the Nox1/4 and the Nox2 inhibitor significantly reduced the non-NO non-prostanoid relaxations (Fig. 11A, B; Table 2) and blunted the NADPH-stimulated generation of  $\text{O}_2^{\cdot-}$  (Fig. 11C), thus suggesting that Nox4 and Nox2-derived ROS are involved in the endothelium-dependent relaxations of human intrarenal arteries.

## 4. Discussion

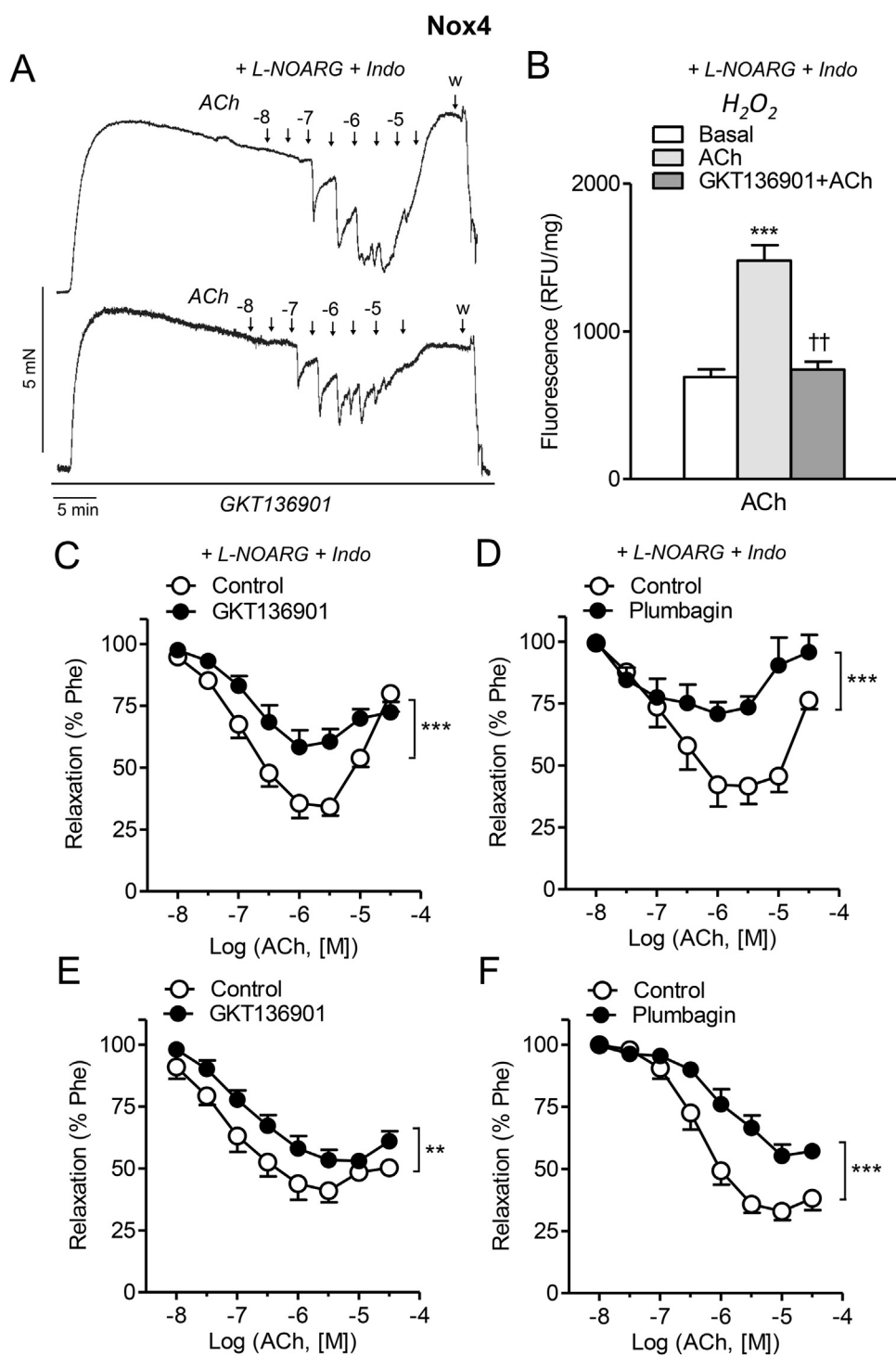
NADPH oxidase-derived oxidative stress has emerged as a critical pathogenic factor in the development of diabetes microvascular complications, and newly developed Nox- inhibiting drugs are currently being investigated as renoprotective therapeutic tools to ameliorate diabetic nephropathy [14,26]. However, the functional role of Nox



**Fig. 5.** Nox4 inhibitors reduce NADPH-stimulated  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  production in rat renal vascular and cortical tissues. (A, B) Effects of the Nox1/4 inhibitor GKT136901 (0.1  $\mu\text{M}$ ) and the non-selective Nox inhibitor plumbagin (1  $\mu\text{M}$ ) on the NADPH-stimulated levels of  $\text{O}_2^{\cdot-}$  (A) and  $\text{H}_2\text{O}_2$  (B) production measured by lucigenin-enhanced chemiluminescence and by Amplex Red fluorescence, respectively, in renal arteries and cortex from rat kidney. Results are expressed in counts per minute (cpm) per mg of tissue for chemiluminescence and in relative fluorescence units (RFU) per mg of tissue for fluorescence. Bars represent mean  $\pm$  SEM of 8–10 arterial or cortical samples from 4–5 animals. Significant differences between means were analyzed using one-way ANOVA followed by Bonferroni as a *posteriori* test  $^{***}P < 0.001$  versus basal levels,  $^{\dagger}P < 0.01$   $^{\dagger\dagger}P < 0.01$  versus NADPH-stimulated.

enzymes in renal vasculature and hemodynamics remains unclear, and ROS have recently been shown to contribute to the endothelial vasodilatation of human and rat intrarenal arteries [18,22]. Therefore, Nox enzymes were localized in the renal vascular wall and Nox2- and Nox4-mediated ROS generation and its contribution to the endothelial-dependent vasodilatation were assessed in human and rat renal arterioles. Our data first provide evidence that NADPH oxidases, namely Nox2 and Nox4, are present in the renal endothelium and represent relevant sources of vasodilator  $\text{H}_2\text{O}_2$  under physiological conditions.

In the present study, intrarenal arteries generated basal and NADPH-stimulated  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$ , and ROS vascular production was associated to the inhibitory effect of the ROS scavenger tempol and of

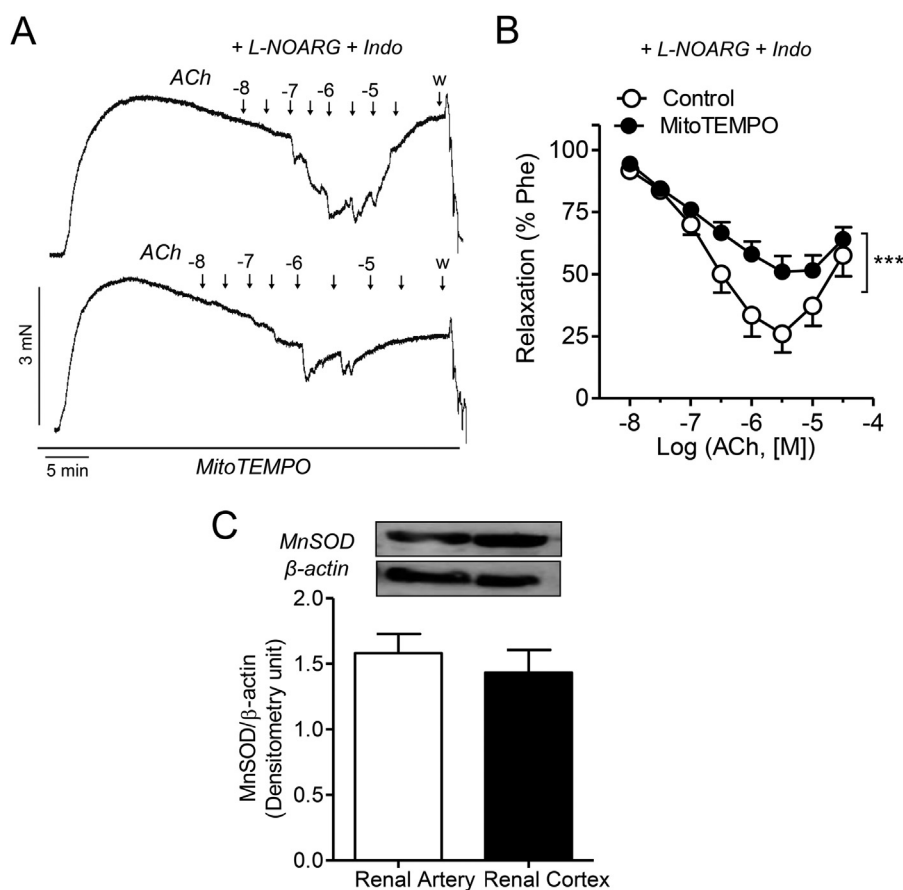


**Fig. 6.** Nox4 inhibitors reduce non-NO non-prostanoid endothelium-dependent relaxations of rat renal interlobar arteries. (A) Representative traces of a rat interlobar artery with an effective lumen diameter  $l_1 = 300 \mu\text{m}$  and the inhibitory effect of GKT136901 (0.1  $\mu\text{M}$ ) (lower panel) on the relaxations induced by acetylcholine (ACh) (upper panel) in arteries precontracted with Phe and treated with L-NOARG (100  $\mu\text{M}$ ) and indomethacin (0.3  $\mu\text{M}$ ) (upper panel). (B) Effect of GKT136901 (0.1  $\mu\text{M}$ ) on  $\text{H}_2\text{O}_2$  production stimulated by ACh (10  $\mu\text{M}$ ) in rat isolated interlobar arteries measured by Amplex Red fluorescence. Results are expressed in relative fluorescence units (RFU) per mg of tissue. Bars represent mean  $\pm$  SEM of 4 animals. (C–F) Average inhibitory effect of the Nox1/4 GKT136901 inhibitor (0.1  $\mu\text{M}$ ) (C, E) and the proposed Nox4 inhibitor plumbagin (1  $\mu\text{M}$ ) (D, F) on the relaxations induced by the endothelial agonist ACh under conditions of NOS and COX blockade (C, D) or in the absence of L-NOARG and indomethacin (Indo) (E, F). Data are shown as the mean  $\pm$  SEM of 8–11 arteries from 5 animals. Significant differences between means were analyzed by paired Student *t*-test. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  versus control before treatment (C–F) and one-way ANOVA followed by Bonferroni as a *posteriori* test \*\* $p < 0.001$  versus basal levels, †† $p < 0.01$  versus ACh-stimulated (B).

the general Nox inhibitor apocynin on the endothelium-dependent relaxations of renal arteries. While CYP epoxygenases have recently been identified as important sources of relaxant  $\text{H}_2\text{O}_2$  in rat intrarenal arteries [22], the inhibitory effect of apocynin first provides evidence for an involvement of NADPH oxidase-derived relaxant ROS in human endothelium-mediated renal vasodilatation, wherein ROS had earlier been proposed to play a role [18]. As shown for rat intrarenal arteries,  $\text{H}_2\text{O}_2$ -mediated vasodilatation sensitive to apocynin was unmasked under conditions of NOS and COX blockade, since both NO and prostacyclin are involved in the endothelium-dependent relaxations of intrarenal arteries, while  $\text{H}_2\text{O}_2$  plays a main role in the renal EDH-type relaxant responses [22]. On the other hand, the inhibitory effect of

apocynin and sulfaphenazole, but not of the NOS inhibitor L-NOARG on the NADPH-stimulated chemiluminescent and fluorescent signals, suggests that Nox contribute along with CYP 2C epoxygenases [22] to the NADPH-dependent  $\text{O}_2^{\cdot -}$  and  $\text{H}_2\text{O}_2$  generation in intrarenal arteries under physiological conditions, while ruling out the contribution of NOS. These results also confirm that NADPH-stimulated chemiluminescent and fluorescent signals in intact arteries reflect ROS generation not only by NADPH oxidases but also by other enzymes such as CYP epoxygenases in the vascular wall, as recently shown in membrane assays [27].

All the NADPH oxidase isoforms –Nox1, Nox2, Nox4 and the human isoform Nox5– are present in kidney tissues including mesangium,



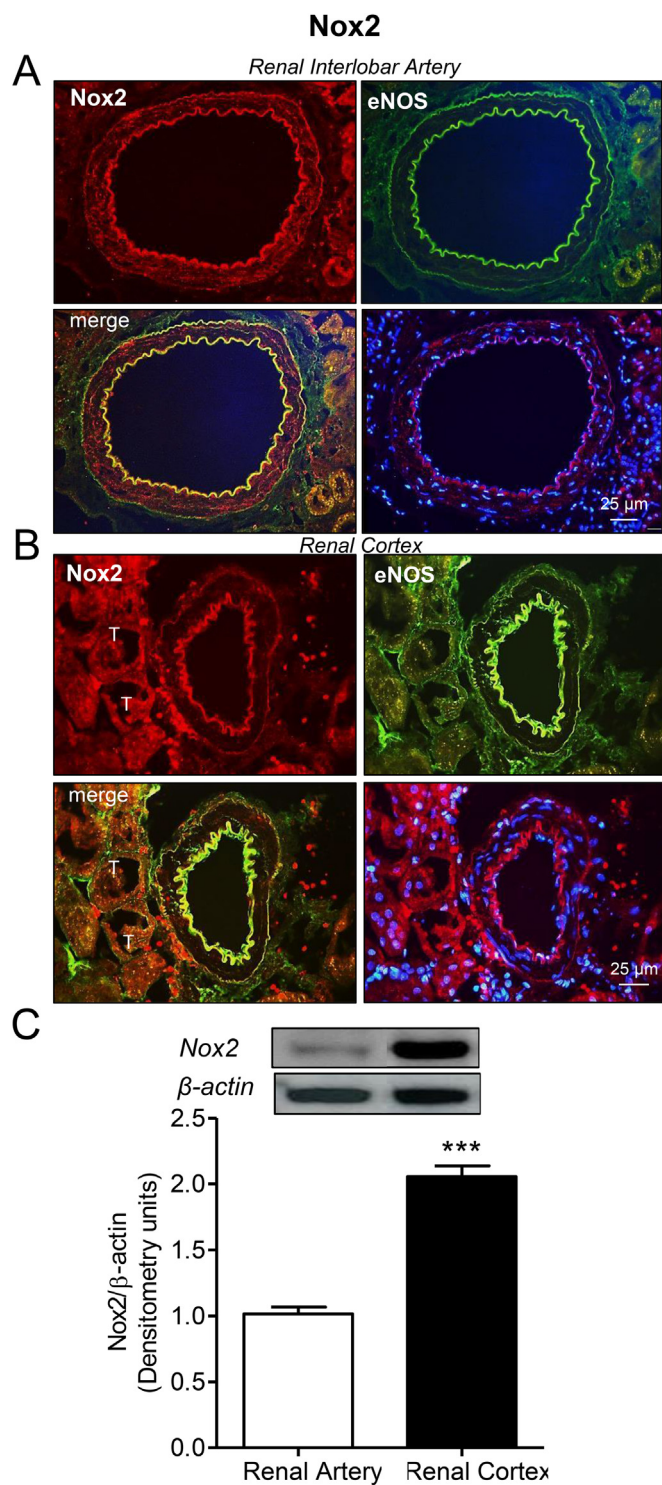
**Fig. 7.** The mitochondrial ROS scavenger MitoTEMPO inhibits the non-NO non-prostanoid endothelium-dependent vasodilation of rat renal interlobar arteries. (A) Representative traces of a rat interlobar artery with an effective lumen diameter  $l_1 = 311 \mu\text{m}$  showing the inhibitory effect of MitoTEMPO ( $1 \mu\text{M}$ ) (lower panel) on the relaxations induced by acetylcholine (ACh) (upper panel) in arteries precontracted with Phe and treated with L-NOARG ( $100 \mu\text{M}$ ) and indomethacin (Indo) ( $0.3 \mu\text{M}$ ) (upper panel). (B) Average inhibitory effect of MitoTEMPO on the non-NO non-prostanoid endothelium-dependent vasodilation of rat intrarenal arteries. Data are shown as the mean  $\pm$  SEM of 8 arteries from 4 animals. (C) Western blot analysis of Mn-SOD expression in samples of renal artery and cortex showing similar protein levels in samples of renal arteries and cortex. Results were quantified by densitometry and presented as a ratio of density of Mn-SOD band versus those of  $\beta$ -actin from the sample. Bars are mean  $\pm$  SEM of 4 animals. Significant differences between means were analyzed by paired Student *t*-test. \*\*\**p* < 0.001 versus control before treatment.

tubules, macula densa, endothelium and VSM [15], and in particular, Nox2 and Nox4 but not Nox1 mRNA, have been identified in intrarenal arteries of the human kidney although their functional significance and their role in renal hemodynamics remain unclear [18].

Nox4 was originally termed *Renox* due to its high expression in the kidney, mainly in the renal tubular system, mesangial cells and podocytes [25,28], and has mostly been associated to oxidative stress in diabetic nephropathy and renal injury [13,19,29,30]. However, there is some controversy about the role of Nox4 in kidney function since data in Nox4-deficient animals do not involve this isoform as a major driver of renal disease [31]. The current findings in renal arteries are in line with the increasing experimental evidence supporting a protective role for Nox4 in the vasculature [23,32–35]. Nox4 beneficial versus adverse vascular effects were initially ascribed to cell-type specific actions, i.e. endothelium versus fibroblasts and VSM. We demonstrate here that Nox4 protein is highly expressed in both intrarenal arteries and cortex of healthy rats, in agreement with data identifying Nox4 mRNA in renal arteries from elderly patients [18]. Double immunolabelling for Nox4 and eNOS revealed that this Nox catalytic subunit is widely distributed in the renal vascular wall, predominantly within the endothelium, and in the renal cortex its presence was not restricted to the tubules but also to the endothelium of arterioles and glomeruli. Nox4 is constitutively active and primarily regulated by changes in gene expression (inducible Nox) [36]. Unlike Nox1 or Nox2 that primarily produce  $\text{O}_2^{\cdot-}$ , Nox4 has been demonstrated to produce  $\text{H}_2\text{O}_2$  rather than  $\text{O}_2^{\cdot-}$  due to a highly conserved E-loop that promotes rapid dismutation of  $\text{O}_2^{\cdot-}$  before it leaves the enzyme [37]. The current results show that NADPH-dependent generation of both  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  was reduced by Nox4 inhibitors in human and rat renal tissues, inhibition of  $\text{H}_2\text{O}_2$  being more pronounced in renal cortex than in arteries, which suggests a differential ROS formation by Nox4 in renal cortical and vascular samples. In this respect, Nox 4 is the most highly expressed Nox homolog in endothelial

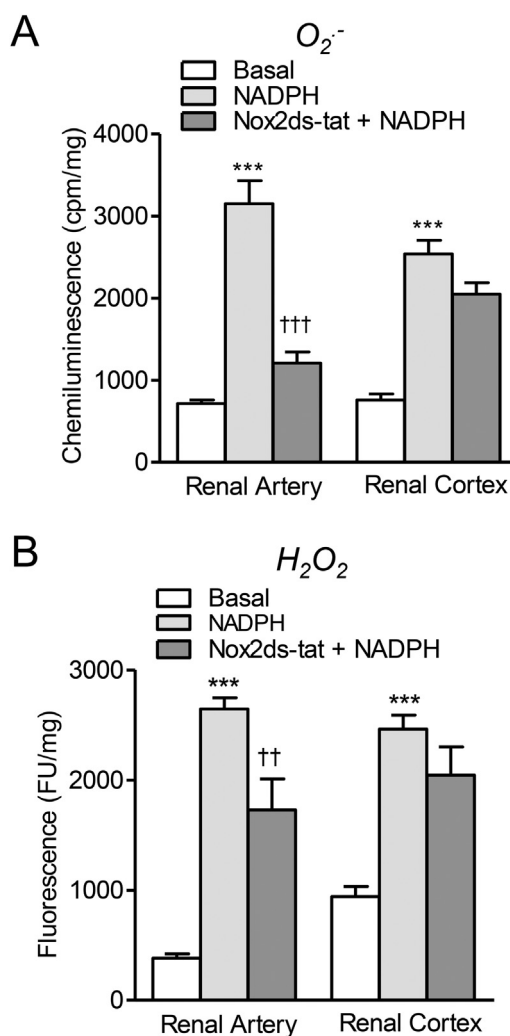
cells, wherein it has been shown to produce high amounts of  $\text{O}_2^{\cdot-}$  anions that are rapidly converted to  $\text{H}_2\text{O}_2$  [38,39], a more stable molecule not inactivating endothelial NO in the vascular wall.

In healthy vascular tissue, Nox4 was initially involved in proliferation of endothelial cells and angiogenesis [32,40], and in VSM differentiation [41]. Moreover, here we demonstrate that Nox4-derived  $\text{H}_2\text{O}_2$  contributes to the endothelium-dependent vasodilation of human and rat intrarenal arteries, since the relaxations and  $\text{H}_2\text{O}_2$  generation elicited by the endothelial agonist ACh were significantly reduced by pharmacological Nox4 inhibition. These findings would therefore support a protective role for Nox4 in the renal vasculature and are consistent with earlier studies showing increased  $\text{H}_2\text{O}_2$  production in endothelial cells, enhanced arterial endothelium-dependent vasodilation and reduced blood pressure in transgenic mice with endothelium-targeted Nox4 overexpression [23]. Likewise, Nox4-deficient mice exhibited reduced endothelial  $\text{H}_2\text{O}_2$  production and endothelial dysfunction [33]. Since Nox4 has been shown to increase NO formation by enhancing eNOS expression and activity through p38MAPK-mediated eNOS phosphorylation [32,42], an involvement of endothelial NO in the Nox4-mediated relaxations of renal arteries would be expected. However, relaxations sensitive to Nox4 inhibitors were also obtained under conditions of NO and prostanoid synthesis blockade suggesting that endothelial Nox4-derived  $\text{H}_2\text{O}_2$  might contribute to renal vasodilation independently of NO and prostacyclin. Contribution of NO to the Nox4-mediated vasodilation might vary depending on the differential stimulation of endothelial Nox4. Thus, Nox4-derived  $\text{H}_2\text{O}_2$  released by laminar shear stress has been reported to activate endothelial NOS phosphorylation and NO production [42], while in our study Nox4-mediated relaxation of intrarenal arteries was stimulated by the endothelial agonist ACh, Nox4 having been shown to be rapidly up-regulated through translational mechanisms upon agonist stimulation [43,44].

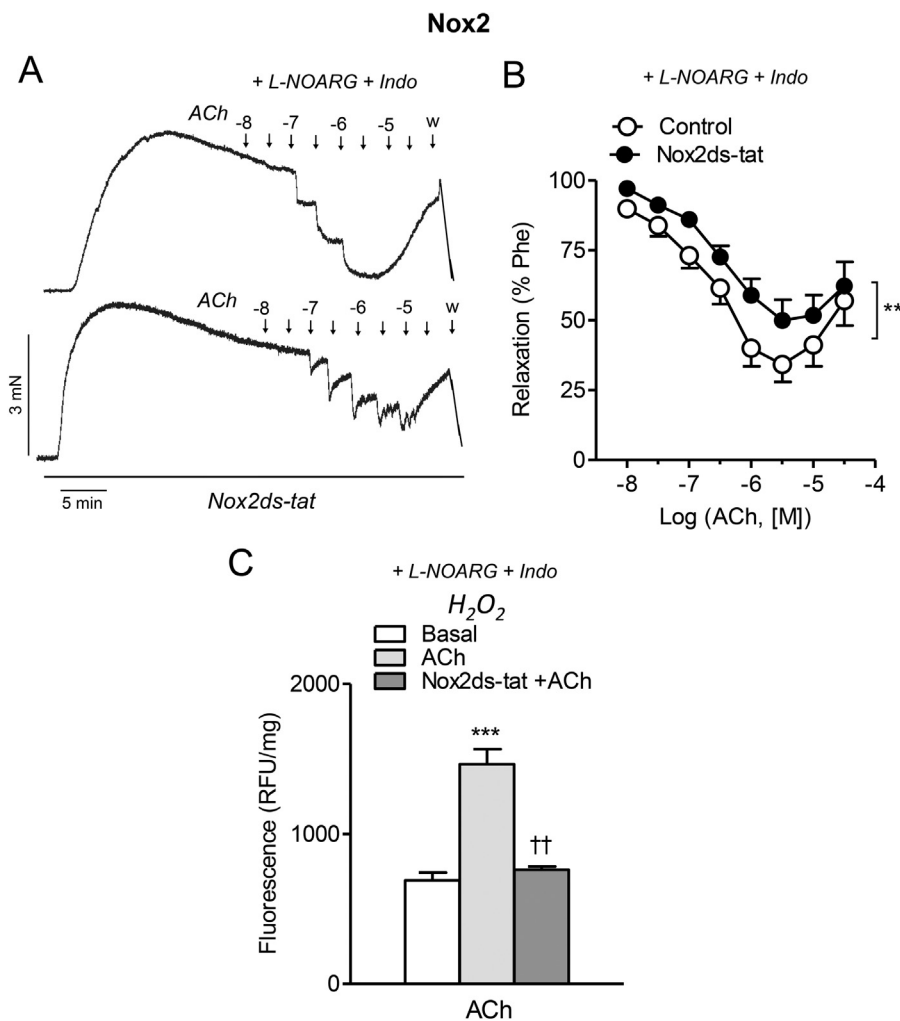


Subcellular localization of Nox4 has been reported to be wide including endoplasmic reticulum (ER), plasma membrane, nucleus and mitochondria [43], and differential functions have been ascribed to the Nox4 protein depending on its intracellular location. Thus, Nox4 in ER of endothelial cells is involved in the regulation of another ER protein, the tyrosine phosphatase 1B (PTP 1B), and in the redox signalling coupled to the epidermal growth factor (EGF) receptor [45]. Nox4 has been identified as a functional source of ROS generation in the mitochondria of kidney cortex, wherein the mitochondrial form of SOD MnSOD effectively dismutates Nox4-derived superoxide to H<sub>2</sub>O<sub>2</sub>

**Fig. 8.** Nox2 is expressed in the endothelium of renal arteries and arterioles colocalized with eNOS. (A, B) Immunohistochemical demonstration of Nox2 in renal interlobar arteries and cortex of rat kidney. Immunofluorescence for Nox2 protein (red areas) was present throughout the endothelial lining of the renal interlobar artery and VSM (A) and in endothelium of the small arterioles and tubules (T) in the renal cortex (B). Endothelial cell layer was visualized with the anti-eNOS marker (green). Immunofluorescence double labelling for eNOS and Nox2 expression demonstrates colocalization in endothelium (yellow areas) in both renal artery and cortex. Scale bars indicate 25 μm. Sections are representative of n = 3 animals. (C) Western blot analysis of Nox2 expression in renal artery and cortex showing higher Nox2 protein levels in samples of renal cortex than in those of isolated renal arteries. Results were quantified by densitometry and presented as a ratio of density of Nox4 band versus those of β-actin from the sample. Data are shown as the mean ± SEM of 4 animals. Significant differences were analyzed using unpaired t-test \*\*\*P < 0.001 versus renal cortex.



**Fig. 9.** Nox2 inhibition reduces NADPH-stimulated O<sub>2</sub><sup>·-</sup> and H<sub>2</sub>O<sub>2</sub> production in rat renal arteries. (A, B) Effects of the Nox2 inhibitor Nox2ds-tat (1 μM) on the NADPH-stimulated levels of O<sub>2</sub><sup>·-</sup> (A) and H<sub>2</sub>O<sub>2</sub> (B) measured by lucigenin-enhanced chemiluminescence and by Amplex Red fluorescence, respectively, in renal arteries and cortex from rat kidney. Results are expressed in counts per minute (cpm) per mg of tissue for chemiluminescence and in relative fluorescence units (RFU) per mg of tissue for fluorescence. Bars represent mean ± SEM of 8 arterial or cortical samples from 4 animals. Significant differences between means were analyzed using one-way ANOVA followed by Bonferroni as a *post-hoc* test \*\*\*P < 0.001 versus basal levels, ††P < 0.01 versus NADPH-stimulated.



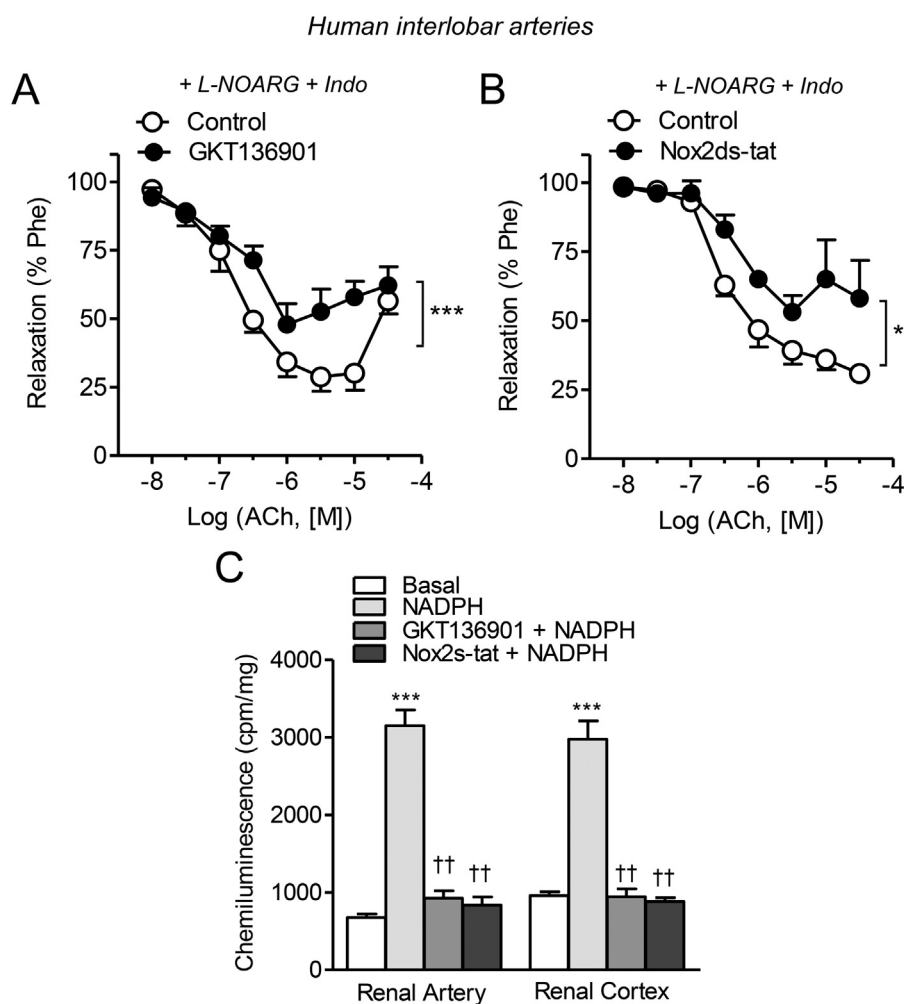
**Fig. 10.** Nox2ds-tat reduces non-NO non-prostanoid endothelial vasodilatation and H<sub>2</sub>O<sub>2</sub> production stimulated by ACh. (A) Representative traces of a rat interlobar artery with an effective lumen diameter  $l_1 = 288 \mu\text{m}$  and the inhibitory effect of Nox2ds-tat (1  $\mu\text{M}$ ) (lower panel) on the relaxations induced by acetylcholine (ACh) (upper panel) in arteries pre-contracted with Phe and treated with L-NOARG (100  $\mu\text{M}$ ) and indomethacin (0.3  $\mu\text{M}$ ) (upper panel). (B) Average inhibitory effect of the Nox2 inhibitor Nox2ds-tat (1  $\mu\text{M}$ ) on the relaxations induced by the endothelial agonist ACh under conditions of NOS and COX blockade (100  $\mu\text{M}$  L-NOARG and 0.3  $\mu\text{M}$  indomethacin, Indo). Data are the mean  $\pm$  SEM of 8 arteries from 4 animals. Significant differences between means were analyzed by paired Student *t*-test. \*\**p* < 0.01 versus control before treatment. (C) Effect of Nox2ds-tat on the H<sub>2</sub>O<sub>2</sub> production stimulated by ACh (10  $\mu\text{M}$ ) in rat isolated interlobar arteries measured by Amplex Red fluorescence. Results are expressed in relative fluorescence units (RFU) per mg of tissue. Bars represent mean  $\pm$  SEM of 3 animals. Significant differences between means were analyzed using one-way ANOVA followed by Bonferroni as a *posteriori* test \*\*\**P* < 0.001 versus basal levels, ††*P* < 0.01 versus ACh-stimulated.

[43,46]. Moreover, mitochondrial Nox4 expression was up-regulated in a rat model of diabetes [46]. The results of the present study suggest a role for mitochondrial Nox4 in the endothelial relaxation of intrarenal arteries, since the mitochondria-targeted antioxidant MitoTEMPO mimicked the inhibitory effect of Nox4 inhibitors on acetylcholine relaxations, and MnSOD expression in renal arteries was as high as in renal cortex suggesting a major role of mitochondrial SOD in H<sub>2</sub>O<sub>2</sub> generation in the vascular wall.

The classic NADPH oxidase gp91phox -Nox2-, initially found in phagocytic cells, is now recognized as an important source of ROS generation also in the vascular wall including the endothelium [39]. Like Nox4, Nox2 has been implicated in renal oxidative stress during diabetes [19,47], although studies in Nox2-deficient mice suggest that Nox2 is not critical for the pathogenesis of diabetic nephropathy [48]. Furthermore, Nox2 is involved in kidney tubular and vascular functions, and participates in the tubule-glomerular feed-back and macula densa responses to high salt, and in the regulation of afferent arteriolar tone, respectively [21,49]. While in afferent arterioles Nox2 is activated by angiotensin II and generates vasoconstrictor O<sub>2</sub><sup>•-</sup> [21], the present results demonstrate that Nox2 is present in the endothelium of renal arteries and arterioles and generates both O<sub>2</sub><sup>•-</sup> and vasodilator H<sub>2</sub>O<sub>2</sub>. Moreover, acetylcholine-stimulated H<sub>2</sub>O<sub>2</sub> generation and endothelium-dependent relaxations of human and rat arterioles were reduced by the selective Nox2 inhibitor Nox2ds-tat first suggesting the involvement of endothelial Nox2-derived vasodilator H<sub>2</sub>O<sub>2</sub> in the kidney. These findings in intrarenal arterioles would be consistent with those earlier reported for human coronary arterioles showing that Nox2 is a relevant source of H<sub>2</sub>O<sub>2</sub> in the endothelium-dependent vasodilatation of the

heart [9]. Previous studies have shown that in VSM Nox2 contributes to angiotensin II-induced vasoconstriction and VSM hypertrophy [50]. We have also found Nox2 distributed in renal VSM, in particular in the larger renal interlobar arteries, whereas Nox2 was mostly found in the endothelium of the smaller cortex arterioles. The different vascular actions mediated by Nox2 activation in renal arteries, i.e. vasodilation versus vasoconstriction/VSM hypertrophy, might therefore be ascribed to differential cell-dependent activation of Nox2 in the endothelium or VSM, respectively, as proposed for human coronary arterioles [9]. However, the ability to induce vasoconstriction or vasodilatation has also been ascribed to the type of Nox isoenzyme, since Nox2-derived O<sub>2</sub><sup>•-</sup> and Nox4-derived H<sub>2</sub>O<sub>2</sub> were recently shown to contract and relax, respectively, renal arterioles thus counterregulating afferent arteriole myogenic response [51]. H<sub>2</sub>O<sub>2</sub> is released upon endothelial stimulation in renal arteries and participates in the EDH relaxation of renal VSM [22]. In the present study, we demonstrate the presence of Nox2 and Nox4 in the endothelium of renal arterioles and glomeruli, and the ability of these isoenzymes to generate both O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>. The lower levels of constitutive catalase in renal arteries compared to cortex, along with the high constitutive expression of both CuZnSOD [22] and MnSOD, as shown in the present study, probably facilitates H<sub>2</sub>O<sub>2</sub> generation and enhances its vasodilator action in the arterial wall.

In conclusion, the results of the current study shed some light on the role of Nox enzymes in the regulation of renal hemodynamics and first provide evidence for endothelial Nox4 and Nox2 as functional enzymatic sources of vasodilator H<sub>2</sub>O<sub>2</sub> in preglomerular human and rat renal arterioles. Involvement of Nox4 in the endothelium-dependent relaxations of human renal arteries adds further support to the



**Fig. 11.** Effect of Nox4 and Nox2 inhibitors on the non-NO non-prostanoid endothelium-dependent relaxations and ROS generation of human intrarenal arteries. (A, B) Average inhibitory effect induced by the Nox1/4 inhibitor GKT136901 (0.1  $\mu$ M) (A) and the Nox2 inhibitor Nox2ds-tat (1  $\mu$ M) (B) on the relaxations elicited by ACh in human intrarenal arteries under conditions of NOX and COX blockade (100  $\mu$ M L-NOARG and 0.3  $\mu$ M indomethacin, Indo). Data represent mean  $\pm$  SEM of 7–9 arteries from 4 patients. Significant differences between means were analyzed by paired Student *t*-test. \**p* < 0.05; \*\*\**p* < 0.001 versus control before treatment. (C) Average effect of GKT136901 and Nox2ds-tat on NADPH-stimulated  $O_2^{\cdot -}$  production in samples of human intrarenal arteries and renal cortex measured by lucigenin-enhanced chemiluminescence. Bars show mean  $\pm$  SEM of 7–10 arterial or cortical samples from 3 patients. Significant differences between means were analyzed using one-way ANOVA followed by Bonferroni as a *posteriori* test \*\*\**p* < 0.001 versus basal levels, ††*p* < 0.01 versus NADPH-stimulated.

increasing experimental evidence showing the protective role of this  $H_2O_2$ -generating Nox subunit in the cardiovascular system, wherein it is involved in the redox signalling of the flow-induced NOS activation and NO release [52], protects the vasculature during ischemic and inflammatory stress and has anti-atherosclerotic functions [33–35]. Moreover, both endothelial and cardiac myocytes Nox4 contribute to protection against chronic hemodynamic overload-induced cardiac remodelling [53]. The vasodilator function shown for the Nox4 and Nox2 catalytic isoforms in the renal vasculature, the beneficial action against kidney fibrosis reported for Nox4 during chronic renal injury [54] and the lack of definitive evidence supporting Nox2 and Nox4 involvement as drivers of diabetic nephropathy in Nox-deficient mice, suggest that other specific Nox catalytic isoforms such as Nox1 might account for the adverse vascular effects of Nox-derived ROS during diabetes and metabolic disease, as recently proposed [55]. Therefore, despite the large body of evidence involving Nox family of NADPH oxidases, in particular Nox4, in the pathogenesis of diabetic nephropathy [13,19,26], caution must be taken when considering Nox2 and Nox4 as potential targets in the treatment of diabetes microvascular complications, and selective inhibition of other Nox catalytic subunits or sources of oxidative stress must be considered as pharmacological therapeutic targets for the treatment of diabetic nephropathy.

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#### Conflict of interest

All the authors declare no conflicts of interest.

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